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Association between arginase-containing platelet-derived microparticles and altered plasma arginine metabolism in polycystic ovary syndrome

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Abstract:

Background: Polycystic ovary syndrome (PCOS) is an endocrine and metabolic disease associated with insulin resistance and increased risk of cardiovascular diseases. However, a biomarker for potential cardiovascular disease in PCOS patients is not available.

Materials and methods: Twenty-two patients with PCOS and 22 healthy controls were included in the present study and amino acid profiling was performed on fasting plasma samples. Circulating microparticles were characterized by FACS analysis and complemented with enzyme activity assays.

Results: The ratio of ornithine to arginine was significantly increased in plasma form PCOS patients and was associated with a significant increase in plasma arginase levels and activity. Platelet-derived microparticles were identified to be the main sources of the increased plasma arginase activity.

Conclusions: Increased levels of arginase-bearing platelet-derived microparticles contribute to the alteration of the arginine metabolism in patients with polycystic ovary syndrome. Moreover, ornithine and arginine levels represent early biomarkers of potential cardiovascular disease in PCOS patients.

Keywords: Arginase, polycystic ovary syndrome, platelet-derived microparticles, ornithine, arginine

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrinopathies in women of reproductive-age with a prevalence of 6-10% [1]. Although the etiology of PCOS is unclear, it is considered to be a multifactorial disease involving genetic, endocrine and metabolic factors [2]. Clinically, the syndrome is characterized by hyperandrogenism, menstrual dysfunction and infertility, but it is also associated with insulin resistance and an increased risk of developing cardiovascular disease [3]. Indeed, PCOS patients demonstrate endothelial and platelet dysfunction [4,5] as well as a pro-thrombotic state [6,7]. However, a biomarker that could predict a risk for cardiovascular disease development in PCOS patients is lacking. Given that plasma free amino acid profiles have been reported to predict the onset of cardiovascular disease [8], the aim of the present study was to perform amino acid profiling to identify possible markers of increased cardiovascular risk in PCOS patients.

Research Design and Methods

Healthy donors and PCOS patients

A total of 22 treatment naïve PCOS patients, diagnosed according to the Rotterdam criteria (mean age 29.34±1.25 years; BMI 26.08±1.38 Kg/m^2, fasting plasma glucose 88.32±1.62, HOMA-IR 2.28±0.35), attending the clinic for fertility problems were included in the present study. Twenty two age-matched female subjects (mean age 30.39±1.27 years; BMI 20.86±0.49 Kg/m^2) without cycle disorder, PCOS or insulin resistance served as the control group. All of the participants were aged between 21-35 year old. None of the participants were smokers, had acute or chronic diseases (except PCOS for the PCOS group), took any medication or any nutritional supplements. The sample size was chosen after power calculation. The study protocol was approved by the ethics committee of the Goethe University Hospital (No. E 61/09 Geschäfts Nr 86/09) and the Landesärztekammer Hessen. All of the participants gave written informed consent.

Platelet isolation and plasma sampling

All participants were fasting at the time of blood sampling. Platelets were obtained by centrifugation (900*g*, 7 minutes) of platelet-rich plasma, as described [9]. The resulting platelet-poor plasma was collected and frozen at -70°C for further evaluation.

Quantification of free amino acids in plasma

Plasma samples (100 µL) were used for amino acid analysis. Sample preparation was performed using the EZ:faast LC-MS free amino acid analysis kit (Phenomenex, Aschaffenburg, Germany) according to the manufacturer's instructions, with minor modifications. Internal standards (10 µL) were applied to all samples and to the standard curve. Analysis of metabolites was performed by LC-MS/MS using the EZ:faast AAA-MS

HPLC column on an Agilent 1290 Infinity LC system (Agilent, Waldbronn, Germany) coupled to a QTrap 5500 mass spectrometer (Sciex, Darmstadt, Germany). Electro spray ionization in positive mode was employed. The intensity of the measured metabolite was normalized to internal standards. Analyst 1.6.2 and MultiQuant 3.0 (Sciex, Darmstadt, Germany), were used for data acquisition and analysis, respectively.

Measurement of arginase activity

Arginase activity was determined in plasma (MAK112, Sigma, Steinheim, Germany) or in platelets (Abcam, Cambridge, UK) using commercially available kits and according to manufacturer's instructions.

Measurement of arginase levels

Arginase levels were measured in plasma using a commercially available ELISA (antibodies-online, Aachen, Germany) and according to manufacturer's instructions.

Flow cytometric analysis of microparticles

Microparticles were measured in plasma from healthy donors and PCOS patients. Plasma samples were stained with the anti-annexin V antibody and CD42a-PE (platelet marker, BD Biosciences, Heidelberg, Germany), CD235a-FITC (erythrocyte marker, Thermofisher, Dreieich, Germany) and asialoglycoprotein receptor 1 (ASGPR1, hepatocyte marker, R&D system, Wiesbaden, Germany). In a different set of experiments, microparticles were isolated from plasma by centrifugation (20.000g, 30 min, 4°C), permeabilized and stained with Alexa Fluor 488-conjugated anti-arginase 1 antibody (Thermofisher, Dreieich, Germany) followed by FITC-conjugated secondary antibody and CD42a-PE. Microparticles were measured on a BD FACSVerse flow cytometer (BD Biosciences, Heidelberg, Germany). The microparticles-gate was determined using Megamix beads (BioCytex, Marseille, France) and microparticles were defined as particles less than 1.0 µm in size and were positive to annexin. Conjugate isotype-matched immunoglobulin was used as a negative control. The results are presented as number of microparticle events in the microparticle-gate pro µL plasma.

Statistical analysis

Data are expressed as mean ± SEM and statistical evaluation was performed with either Student's *t* test or 2-way ANOVA followed by Newman-Keuls post-test where appropriate (GraphPad Prism 7). Values of $P<0.05$ were considered statistically significant.

Results

Arginase activity is increased in PCOS patients

In order to characterize the consequences of PCOS on the metabolic profile, amino acid levels in plasma from PCOS patients were compared with those from healthy controls.

Plasma levels of ornithine were significantly higher in samples from PCOS patients than from healthy controls while arginine levels were significantly lower (**Figure 1A-C**). The altered ratio of ornithine to arginine could reflect a change in the activity of plasma arginase and arginase activity was indeed significantly increased in PCOS plasma (**Figure 1D**). Moreover, the increased arginase activity was associated with enhanced plasma arginase-1 protein levels (**Figure 1E**). Arginase-2 was undetectable (data not shown). Arginase is an intracellular enzyme that can appear in the plasma following cell damage or associated with cell-derived microparticles. Hemolysis and liver damage are known sources of free plasma arginase. However, liver function and free hemoglobin levels were normal in plasma from PCOS patients (**Table 1**) and were comparable with the heathy group.

Platelet-derived microparticles were the main sources of arginase activity

In order to investigate the possible contribution of cell –derived microparticles to circulating arginase activity, microparticles were recovered from the plasma samples, a procedure that significantly decreased plasma arginase levels (**Figure 1F**). These observations suggested that microparticles largely contributed to the increased plasma arginase activity measured in the plasma from PCOS patients. In agreement with the normal liver function and hemolytic rate, numbers of erythrocyte-derived microparticles and hepatocyte-derived microparticles were comparable in the two groups (data not shown). Thus arginase-enriched microparticles in PCOS plasma must originate from an alternative cellular source. Given that the majority of circulating microparticles are derived from platelets and that numbers of circulating platelet-derived microparticles increase in different pathological conditions, including PCOS, the possibility that platelet-derived microparticles might be the source of plasma arginase activity was investigated. Numbers of platelet-derived microparticles were significantly increased in plasma from PCOS patients (**Figure 1G**), and platelet-derived microparticles from PCOS patients demonstrated higher levels of arginase-1 compared to samples from healthy donors (**Figure 1H**). The latter findings were paralleled by significantly higher arginase activity in platelets from PCOS patients versus healthy donors (**Figure 1I**). In order to investigate whether the alteration in arginine metabolism may predict the increased risk of cardiovascular diseases in PCOS patients, we tested whether there was a correlation between the ratio ornithine to arginine and accepted prognostic markers for cardiovascular diseases such as cholesterol, HDL, LDL and triglycerides levels. We found that although levels of these markers were still in the normal range, there was a positive correlation between triglycerides levels and the ratio of ornithine to arginine ($r=0.55$, $p=0.007$).

Discussion

The results of this study indicate that platelets from PCOS patients release high levels of arginase-bearing microparticles which make a significant contribution to the increase in plasma arginase activity and altered arginine metabolism.

Plasma arginine is metabolized by different enzymes including arginine decarboxylase, arginase and nitric oxide (NO) synthases (NOS) to produce urea, ornithine and NO, respectively [10]. Given that arginase and NOS enzymes compete for the same substrate, an increase in plasma arginase activity would be expected to deplete the substrate from the NOS enzymes – and in the case of the endothelial NOS, this would be expected to lead to endothelial dysfunction and the accelerated development of vascular disease [11]. Certainly, increased arginase activity has been associated with the impaired generation of NO in patients with type 2 diabetes [12]. Increased arginase activity may also explain the lack of cardiovascular benefit of arginine supplementation in conditions such as acute myocardial infarction [13].

Arginase is an intracellular enzyme but it has been detected in the plasma following cell damage as well as in cell-derived vesicles that are released in response to cell activation. One important source of plasma arginase is the liver and increased plasma arginase has been associated with liver injury [14,15]. A further cellular source of the enzyme is erythrocytes [16] and neutrophils [17]. Plasma enzymes may bind to plasma proteins or associate with cell-derived microparticles. Interestingly, levels of microparticle-associated arginase activity were significantly enhanced in the plasma from PCOS patients. It is known that the majority of circulating microparticles derive from platelets [18], and numbers of platelet-derived microparticles are enhanced in different pathological conditions, including cardiovascular disease [19] and PCOS [20,21]. Certainly, it was possible to demonstrate that not only were platelet-derived microparticle levels significantly increased in plasma from PCOS patients but the microparticles in question also carried high levels of active arginase. The latter observations would be expected to have a major impact on vascular homeostasis and cardiovascular disease development and reinforce the hypothesis that pre-diabetic PCOS patients are at higher risk of developing cardiovascular disease. All of these observations led to the conclusion that plasma arginase activity and altered plasma levels of arginine and ornithine may be useful as early biomarkers in PCOS patients and indicators of potential cardiovascular disease. Clearly, this proposal would need to be validated in a much larger patient collective as the main limitation of the present study is the low number of participants, also further follow-up studies would be necessary to establish the predictive values of these biomarkers for cardiovascular disease development.

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Author Contributions. H.H. and A.K. performed experiments, acquired the data and analyzed results, S.Z. performed the amino acid profiling, A.W.M. and I.D. characterized the patients, V.R. conceived and designed the research and wrote the manuscript and I.F. revised and edited the manuscript.

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Figure legends

Figure 1. Role of platelet-derived microparticles in the alteration of arginine metabolism and arginase activity in samples from PCOS patients. Levels of ornithine **(A**), arginine (**B**), and the ratio of ornithine to arginine (**C**) in plasma from healthy donors or from PCOS patients, n= 22 (Student's *t* test). (**D**) Arginase activity in plasma from healthy donors or from PCOS patients, n=16 (Student's *t* test). (**E**) Levels of arginase-1 in plasma from healthy donors or from PCOS patients, n=16 (Student's *t* test). (**F**) Levels of arginase-1 in plasma from healthy donors or from PCOS patients in the presence or in the absence of microparticles, n=16 (ANOVA and Newman-Keuls post-test). (**G**) Levels of platelet-derived microparticles (PMPs) in plasma from healthy donors or from PCOS patients, n=17 (Student's *t* test). (**H**) Levels of arginase-1 measured by flow cytometry in PMPs from healthy donors and from PCOS patients, n=17. (**I**) Arginase activity in platelets from healthy donors or from PCOS patients, n=16 (Student's *t* test). *P<0.05, **P<0.01, ***P<0.001.

Table 1. Blood count, hemolysis rate, liver and kidney functions in PCOS patients. Hb, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; γ-GT, γ glutamyl-transferase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AP, alkaline phosphatase.

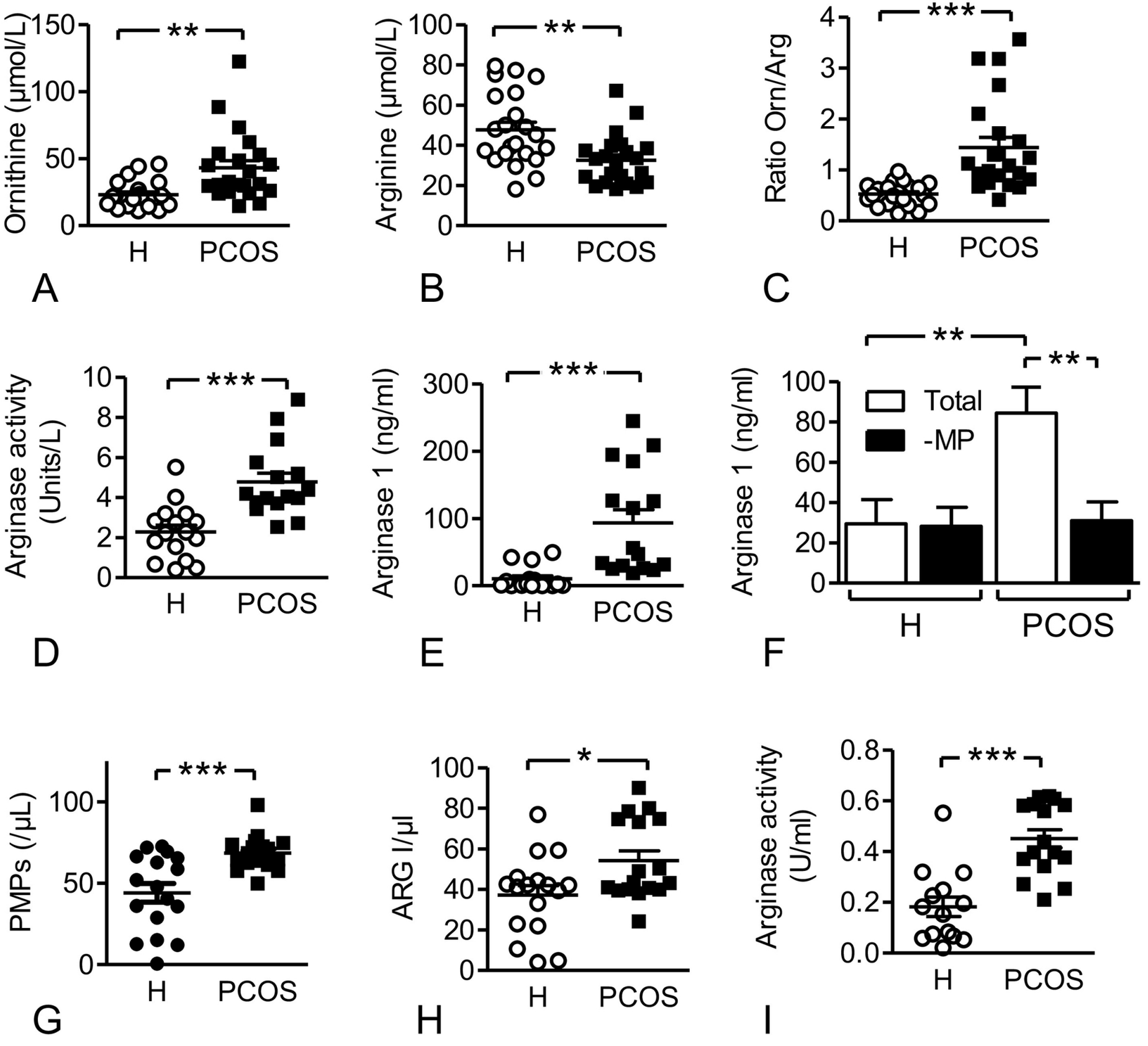
Table: Blood count, hemolysis rate, liver and kidney functions in PCOS patients. Hb, hemoglobin; MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration; MCV, Mean Corpuscular Volume; γ-GT, γ Glutamyl-Transferase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AP, alkaline phosphatase.

	Normal range (Healthy donors)	**PCOS patients**
Erythrocytes (T/l)	3,9-5,3	4,71 ± 0,06
Leukocytes (G/L)	3,98-10,04	6,58 ± 0,37
Platelets (G/L)	150-400	314,21 ± 11,16
Hb (g/dl)	12-16	13,47 ± 0,15
Hematocrite (Vol %)	36-47	40,05 ± 0,74
MCH (pg)	28-32	28,71 ± 0,29
MCHC (g/dl)	30-36	33,21 ± 0,21
MCV (fl)	80-96	86,50 ± 0,87
Liver function		
γ-GT (U/L)	0-40	16,16 ± 1,53
AST (U/L)	0-35	22,55 ± 1,03

ALT (U/L)	0-35	24,11 ± 1,79
AP (U/L)	35-104	63,72 ± 2,34
Kidney function		
Creatinine (mg/dl)	0-0,9	0,73 ± 0,02

Highlights

1- Levels of platelet-derived microparticles are increased in PCOS patients
2- Platelet-derived microparticles carry highly active arginase
3- The ratio ornithine to arginine and arginase activity are increased in plasma from PCOS patients


Ornithine (μmol/L)
150
100
50
0
**
H
PCOS
A
Arginine (μmol/L)
100
80
60
40
20
0
**
H
PCOS
B
Ratio Orn/Arg
4
3
2
1
0
***
H
PCOS
C
Arginase activity (Units/L)
10
8
6
4
2
0
***
H
PCOS
D
Arginase 1 (ng/ml)
300
200
100
0
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H
PCOS
E
Arginase 1 (ng/ml)
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H
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ARG I/μl
100
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Arginase activity (U/ml)
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Figure 1